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# Papers

# *MDR*1 Gene Expression and Prognostic Factors in Primary Breast Carcinomas

**Josef Wallner, Dieter Depisch, Martina Hopfner, Karin Haider, Jürgen Spona, Heinz Ludwig and Robert Pirker**

**To prospectively assess the role of the *MDR*1 gene in breast carcinomas, *MDR*1 RNA levels of breast carcinoma specimens were determined by slot blot analysis. In 59 evaluable patients with primary breast carcinomas, *MDR*1 RNA levels of the carcinomas were negative in 54%, low in 29% and high in 17% of the patients. No differences in age, menopause status, oestrogen and progesterone receptor levels, tumour size, lymph node involvement and c-*erb*B-2/*neu* gene expression were observed between *MDR*1 RNA negative patients and *MDR*1 RNA positive patients.**
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## INTRODUCTION

DRUG RESISTANCE remains an important problem in the chemotherapy of breast carcinomas. Knowledge of the mechanisms involved in this resistance should help to devise ways for overcoming resistance and thus improve treatment outcome.

One mechanism of resistance, multidrug resistance (MDR1), has been extensively studied in cell lines where it is due to the expression of the *MDR*1 gene and of its 170 kD protein product, P-glycoprotein [1–4]. This transmembrane protein functions as an energy-dependent drug efflux pump for anthracyclines, vinca alkaloids and other hydrophobic natural compounds [4]. P-glycoprotein is also expressed in several normal human tissues (e.g. colon and kidney) where it most likely functions as a transport protein [1, 5, 6]. *MDR*1 gene expression was also observed in leukaemias as well as in some solid tumours, particularly in those arising from organs that normally express P-glycoprotein, and was assumed to be involved in clinical drug resistance of these malignancies [1, 6–13]. Since anthracyclines, which are among the most active single agents for the treatment of breast cancer, are affected by the *MDR*1 gene, presence of a functionally active *MDR*1 gene should affect response to anthracyclines and, therefore, might have important therapeutic consequences. This prompted us to prospectively determine both frequency and intensity of *MDR*1 gene expression in breast carcinomas and to assess its association with known prognostic factors including c-*erb*B-2/*neu* gene expression. The results of this study are reported here.

## PATIENTS AND METHODS

*Patients*

From early 1988 to spring 1990, breast carcinoma specimens were obtained from 81 female patients who underwent surgery for breast cancer at the Surgical Department of the General Hospital of Wr. Neustadt, Austria. Tumour specimens were stored at −70°C until use.

*Cell lines*

Drug-sensitive KB-3-1 cells and multidrug-resistant KB-8-5 cells (kindly provided by Dr I. Pastan, NIH, Bethesda) were grown in Dulbecco's modified Eagle's medium, fetal bovine serum, penicillin–streptomycin and glutamine. KB-8-5 cells were cultivated in the presence of 10 ng/ml colchicine.

*MDR*1 RNA levels of tumour specimens and cell lines were determined by a slot blot technique as described [12]. Cells and tumour specimens were homogenised in RNAzol (Cinna/Biotecx, Friendswood, Texas). Total RNA was isolated by acid guanidine thiocyanate–phenol–chloroform extraction [14] using RNAzol as described by the manufacturer. The intactness of the RNA was confirmed by agarose formaldehyde gel electrophoresis [10].

RNA was blotted onto nylon membrane filters. After fixation by UV-irradiation, filters were prehybridised in 50% formamide, 5 × saline–sodium citrate buffer (SSC), 50 mmol/l sodium phosphate buffer (pH 6.5), 5 × Denhardt's reagent and 0.2 mg/ml salmon sperm DNA at 42°C for at least 4 h. A *MDR*1 cDNA (probe 5A; kindly provided by Dr Ira Pastan and Dr Michael Gottesman, National Cancer Institute, NIH, Bethesda) was radiolabelled by means of a random primed DNA labelling kit (Boehringer Mannheim). Hybridisation with the radiolabelled *MDR*1 cDNA was performed in 50% formamide, 5 × SSC, 20 mmol/l sodium phosphate buffer (pH 6.5), 10% dextran sulphate, 1 × Denhardt's reagent and 0.2 mg/ml salmon sperm DNA at 42°C for 18–20 h. Washings with 1 × SSC and 0.1% sodium dodecyl sulphate (SDS) at room temperature (4 × 15 min) were followed by washes with 0.2 × SSC and 0.1% SDS at 55°C (2 × 30 min). Autoradiographic exposures lasted for 2–5 days. Signals were compared to those from drug-sensitive KB-3-1 and multidrug-resistant KB-8-5 cells (Fig. 1). The signal in KB-3-1 cells was negative. An arbitrary value of 30 units (U) was assigned to the *MDR*1 RNA expression of 10 μg of total RNA from KB-8-5 cells [10, 12]. RNA loading was normalised to actin expression.

*Expression of the c-*erb*B-2/*neu *gene*

Expression of the c-*erb*B-2/*neu* gene was determined by measuring its corresponding RNA levels by slot blot analysis. A 40 base single-stranded synthetic oligonucleotide [c-*erb*B-2/*neu* (Pr-2); Oncogene Science] was used for hybridisation. The probe was radiolabelled with the help of a DNA 5'-end labelling kit from Boehringer Mannheim, as described by the manufacturer.

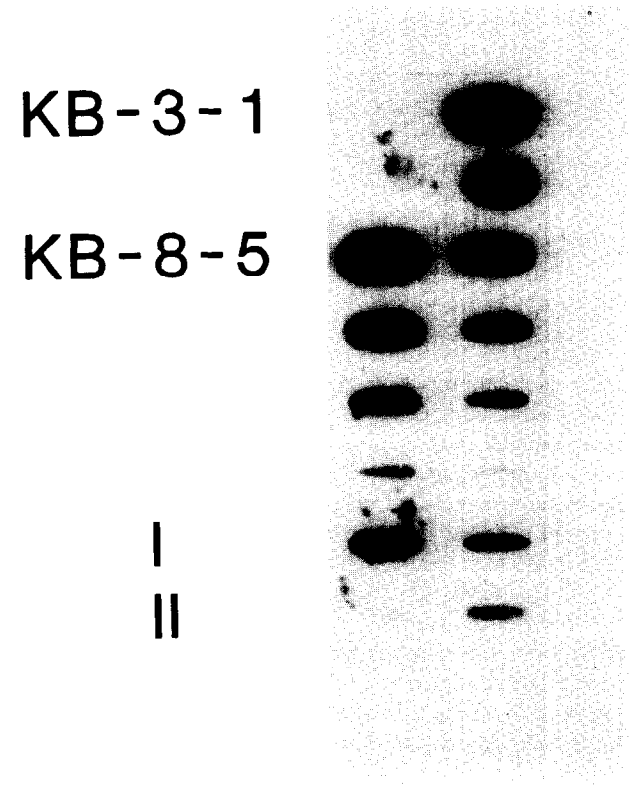


**Fig. 1. Slot blot analysis for *MDR*1 RNA. *MDR*1 RNA levels (left lane) of drug-sensitive KB-3-1 cells (10 and 3 μg RNA), multidrug resistant KB-8-5 cells (10, 3, 1 and 0.3 μg RNA) and breast carcinoma specimens (I, II) were determined. Actin expression (right lane) was used to compensate for the different amounts of RNA loaded.**

Hybridisation was performed in 1 mol/l NaCl, 50 mmol/l Tris-HCl (pH 7.5), 10% dextran sulphate, 1% SDS and 100 μg/ml salmon sperm DNA at 65°C for 16 h. Washings were as described by the manufacturer.

*Determination of oestrogen and progesterone receptors*

Oestrogen and progesterone receptors were determined by the dextran-coated charcoal method as described [15].

*Statistical analysis*

Frequencies were tested by $\chi^2$ tests.

## RESULTS

In order to determine the expression of the *MDR*1 gene, breast carcinoma specimens were obtained at surgery from 81 patients. Determination of *MDR*1 RNA levels was possible in 61 specimens, whereas in 20 specimens *MDR*1 RNA analysis could not be performed because the isolated RNA was either degraded or insufficient for further analysis. 59 of the evaluable specimens were from primary carcinomas and 2 specimens were from local relapses.

*MDR*1 RNA levels of tumour specimens were determined by slot blot analysis and compared with the signals obtained from drug-sensitive KB-3-1 and 4 to 6-fold multidrug-resistant KB-8-5 cells (Fig. 1). *MDR*1 RNA levels were negative in 54% and positive in 46% of the primary carcinomas with low levels (up to 9 U) in 29% and high levels (> 9 U) in 17% of the specimens (Table 1). No expression of the *MDR*1 gene was seen in the 2 specimens obtained from local relapses (data not shown).

Correspondence to R. Pirker, Clinic for Internal Medicine I, Währingergürtel 18–20, 1090 Vienna, Austria.
J. Wallner and R. Pirker are at the First Medical Clinic, Lazarettgasse 14, 1090 Vienna; J. Wallner is also at the Second Medical Clinic, Vienna; D. Depisch and K. Haider are at the Surgical Department, General Hospital, Wr., Neustadt; M. Hopfner and H. Ludwig are at the Second Medical Clinic, Vienna; and J. Spona is at the Department of Molecular Endocrinology, First Clinic of Gynecology and Obstetrics, and Ludwig Boltzmann Institute for Prenatal and Experimental Genome Analysis, Vienna, Austria.
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*Table 1. MDR1 gene expression in breast carcinomas*

| *MDR1* RNA levels | Patients (%) |
|---|---|
| Negative | 32 (54%) |
| Positive | 27 (46%) |
| Low | 17 (29%) |
| High (> 9 U) | 10 (17%) |

The clinical data of the patients with primary breast carcinomas are summarised in Table 2. The patients, aged 34–86 years (median 60), included 13 premenopausal and 46 postmenopausal women. With regard to age or menopause status, however, no significant difference in the frequency of *MDR1* gene expression was observed. Histology and size of primary tumours were not different between *MDR1* RNA-negative and *MDR1* RNA-positive patients. Positive lymph nodes were present in 47% of the *MDR1* RNA negative patients as compared to 52% of the positive patients (not significant). Expression of the *MDR1* gene was not detected in the primary tumours of the 3 patients who had metastatic disease (Table 2). Oestrogen receptors were detected in 66% and progesterone receptors in 77% of the primary carcinomas but no significant difference in receptor positivity was seen between *MDR1* RNA-negative and *MDR1* RNA-positive tumours (Table 3).

*Table 2. MDR1 RNA levels and clinical data of the patients*

| | All patients | *MDR1* negative | *MDR1* positive | | |
|---|---|---|---|---|---|
| | | | Total | Low | High |
| *n* | 59 | 32 | 27 | 17 | 10 |
| Age (yr) | | | | | |
| Median | 60 | 61 | 59 | 56 | 69 |
| Range | 34–86 | 40–83 | 34–86 | 34–81 | 44–86 |
| Menopausal status | | | | | |
| Premenopausal | 13 (22%) | 6 (19%) | 7 (26%) | 6 | 1 |
| Postmenopausal | 46 (78%) | 26 (81%) | 20 (74%) | 11 | 9 |
| Histology | | | | | |
| Ductal adenocarcinoma | 92% | 94% | 89% | 88% | 90% |
| Lobular carcinoma | 8% | 6% | 11% | 12% | 10% |
| Tumour size | | | | | |
| T1 | 8 (13.5%) | 4 (13%) | 4 (15%) | 3 | 1 |
| T2 | 38 (64%) | 23 (72%) | 15 (56%) | 9 | 6 |
| T3 | 8 (13.5%) | 3 (9%) | 5 (18.5%) | 4 | 1 |
| T4 | 1 (2%) | — | 1 (4%) | 1 | — |
| TX | 4 (7%) | 2 (6%) | 2 (7%) | — | 2 |
| Lymph nodes | | | | | |
| N0 | 28 (47.5%) | 17 (53%) | 11 (41%) | 6 | 5 |
| N1 | 22 (37%) | 12 (38%) | 10 (37%) | 9 | 1 |
| N2 | 7 (12%) | 3 (9%) | 4 (15%) | 2 | 2 |
| NX | 2 (3.5%) | — | 2 (7%) | — | 2 |
| Metastasis | | | | | |
| M0 | 56 (95%) | 29 (91%) | 27 (100%) | 17 | 10 |
| M1 | 3 (5%) | 3 (9%) | — | — | — |

All comparisons not significant.

*Table 3. Association between MDR1 RNA levels and oestrogen receptors and progesterone receptors*

| Patients | ER− | ER+ | PR− | PR+ |
|---|---|---|---|---|
| Total (*n*=56) | 19 (34%) | 37 (66%) | 13 (23%) | 43 (77%) |
| *MDR1* RNA-negative (*n*=31) | 10 (32%) | 21 (68%) | 8 (26%) | 23 (74%) |
| *MDR1* RNA-positive (*n*=25) | 9 (36%) | 16 (64%) | 5 (20%) | 20 (80%) |
| Low (*n*=16) | 5 (31%) | 11 (69%) | 3 (19%) | 13 (81%) |
| High (*n*=9) | 4 (44%) | 5 (56%) | 2 (22%) | 7 (78%) |

ER=oestrogen receptors, PR=progesterone receptors. All comparisons not significant.

Expression of the c-*erb*B-2/*neu* gene occurred in 33% of the patients which is consistent with a recent report [16]. However, c-*erb*B-2/*neu* RNA levels were independent of *MDR1* RNA levels (Table 4).

## DISCUSSION

Our prospective study demonstrated the presence of elevated *MDR1* RNA levels in 46% of primary breast carcinomas with high levels in 17% of the specimens. These results are consistent with a recent report on *MDR1* gene expression in 25 out of 49 primary breast carcinomas [17] and similar to a previous study in which 15% of breast carcinomas had *MDR1* RNA levels between 2 and 29 U [10]. However, our results are in contrast to the findings reported by Merkel *et al.* [18] who, employing northern blot analysis, did not detect elevated *MDR1* RNA levels in primary breast carcinomas.

Determination of *MDR1* gene expression can be performed by either measuring *MDR1* RNA levels or by employing immunohistochemical techniques. In our study, *MDR1* RNA levels were determined by slot blot analysis. This technique is highly sensitive and allows a semiquantitative determination of *MDR1* RNA levels. However, a shortcoming of *MDR1* RNA analysis of homogenised tumour samples is that it does not detect any potential heterogeneity with regard to *MDR1* gene expression within the tumour, that contamination with normal cells might affect *MDR1* RNA levels and that sometimes the isolated RNA is degraded or insufficient for further analysis. Using immunohistochemistry techniques, other investigators demonstrated the presence of P-glycoprotein in various percentages of breast carcinomas [19, 20]. Because *MDR1* RNA levels might not be matched by protein expression and/or P-glycoprotein

*Table 4. MDR1 RNA levels and c-erbB-2/neu expression*

| *MDR1* RNA levels | c-*erb*B-2/*neu* negative | c-*erb*B-2/*neu* positive | | |
|---|---|---|---|---|
| | | All | Low | High |
| All patients | 67% | 33% | 30% | 3% |
| Negative (*n*=30) | 70% | 30% | 27% | 3% |
| Positive (*n*=27) | 63% | 37% | 33% | 4% |
| Low (*n*=17) | 47% | 53% | 47% | 6% |
| High (*n*=10) | 90% | 10% | 10% | 0 |

might not be functional, further studies are required to determine whether *MDR*1 RNA analysis, the immunohistochemical technique or a functional assay is better suitable for clinical applications.

We did not observe an association of *MDR*1 gene expression with known prognostic parameters, such as hormone receptor levels, axillary lymph node involvement or c-*erb*B-2/*neu* gene expression (Tables 2–4). However, absence of such an association does not exclude the possibility that patients with detectable *MDR*1 gene expression have a poorer prognosis as compared to patients with no expression. Further follow-up of our patients will clarify the prognostic significance of elevated *MDR*1 RNA levels.

The expression of the *MDR*1 gene in primary tumours supports the hypothesis that drug-resistant carcinoma cells are present already in untreated tumours and subsequently selected by chemotherapy rather than induced by chemotherapy. In acute myeloid leukaemia, elevated *MDR*1 RNA levels in the leukaemic cells at diagnosis were associated with lower remission rates and shorter disease-free as well as overall survival [12]. Recently, human breast carcinoma cells with high *MDR*1 RNA levels were found to require higher doxorubicin concentrations for inhibition of *in vitro* cell growth as compared to cells with low *MDR*1 RNA levels [17]. Therefore, it is likely that expression of the *MDR*1 gene in breast cancer cells also affects tumour response of breast carcinoma patients to drugs that bind to P-glycoprotein (such as anthracyclines and mitoxantrone). Although it is intriguing to speculate that, in particular, patients with high *MDR*1 RNA levels are resistant to these drugs, further studies are required to either prove or reject this hypothesis. Nevertheless, presence of *MDR*1 gene expression in tumour cells should be considered in the chemotherapy of breast carcinomas, particularly in the planning of future adjuvant chemotherapy protocols. Patients expressing the *MDR*1 gene in their tumour cells might be either treated with drugs not affected by the *MDR*1 phenotype (e.g. cyclophosphamide and 5-fluorouracil) or admitted to clinical trials in which adjuvant chemotherapy is combined with reversing agents, such as verapamil [21] or its analogues [22]. Such trials to overcome multidrug resistance should also further define the clinical significance of *MDR*1 gene expression in primary breast carcinomas.


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